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Nonsense-mediated translational repression involves exon junction complex downstream of premature translation termination codon

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ABSTRACT

Human transforming growth factor-β receptor type 2 (TGFβR2) mRNA harboring a premature translation termination codon (PTC) generated by frameshift mutation is targeted for nonsense-mediated translational repression (NMTR), rather than nonsense-mediated mRNA decay (NMD). Here we show that exon junction complex (EJC) downstream of a PTC plays an inhibitory role in translation of TGFβR2 mRNA. Translational repression by core EJC components occurs after formation of 80S ribosome complex, which is demonstrated using different types of internal ribosome entry sites (IRESes). Our findings implicate EJCs or core EJC components as negative regulators of translation.

1. Introduction

Mammalian cells have developed various mechanisms to ensure mRNA quality. Most mRNAs harboring premature termination codons (PTCs) are recognized and downregulated in abundance by nonsense-mediated mRNA decay (NMD) [1,2]. NMD involves the key NMD factors, Upf1, Upf2, and Upf3/3X, and an exon junction complex (EJC) downstream of PTC. The EJC, which is deposited on mRNAs as a consequence of a splicing event, is a multiprotein complex that contains core complex (eIF4AIII, MAGOH, Y14, and Barentsz/MLN51) and other accessory proteins (reviewed in Refs. [1,2] and references therein).

Although most PTC-containing mRNAs are subject to NMD, some PTC-containing transcripts escape NMD and instead are subject to translational repression, which is referred to as nonsense-mediated translational repression (NMTR) [3]. For instance, the transforming growth factor-β receptor type 2 (TGFβR2) mRNA harboring a PTC generated by frameshift mutation in high microsatellite instability (MSI)-H tumors is not targeted for NMD, even when the PTC is located sufficiently upstream of the last exon–exon junction [3]. Instead, this NMD-escaped TGFβR2 mRNA is inhibited at the level of translation. As with NMD, NMTR requires introns downstream of PTC [3], suggesting that either a splicing event downstream of PTC or EJC deposition as a consequence of splicing may contribute to NMTR. Notably, NMTR does not require the key NMD factors Upf1 and Upf2 [3]. The detailed molecular mechanism by which PTC-containing mRNAs are targeted for NMTR rather than NMD remains to be clarified.

To gain molecular insight into NMTR, we characterize the role of EJC downstream of a PTC in translation of PTC-containing TGFβR2 mRNA. Downregulation of the core EJC component eIF4AIII derepresses the translation of PTC-containing TGFβR2 mRNAs. The translational repression by core EJC components is independent of Upf1 function. We also show that NMTR occurs after formation of 80S ribosome complex. All these findings suggest that NMTR of PTC-containing mRNA is mediated by EJC downstream of PTC.

2. Results

2.1. Splicing event or subsequent EJC deposition downstream of PTC is enough to trigger NMTR

To clarify the *cis*-acting elements or *trans*-acting factor(s) for NMTR and to monitor the efficiency of NMTR more quantitatively, chemiluminescence-based reporter plasmids were constructed as follows (Fig. 1A). The best-characterized NMTR-targeted mRNA is TGFβR2 mRNA [3]. Thus, complementary DNA (cDNA) or genomic DNA (gDNA) sequence corresponding to the 3′-untranslated region

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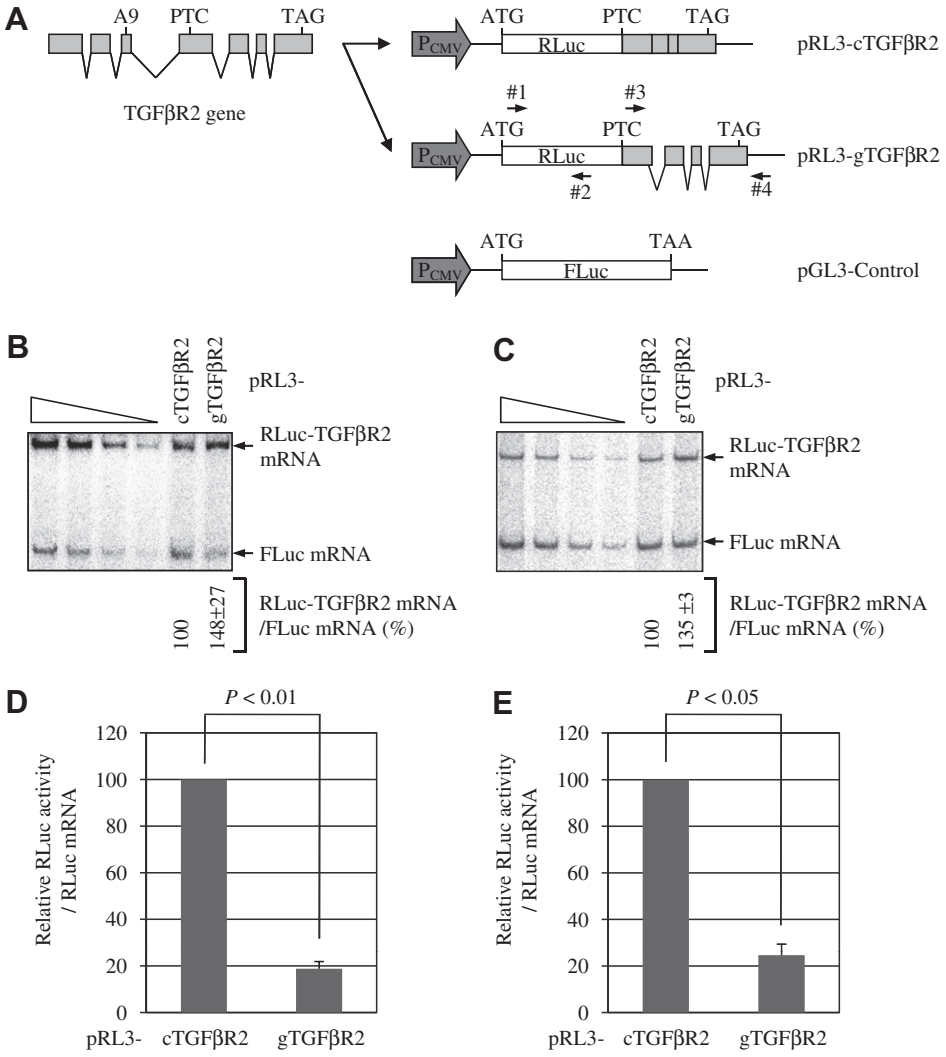

Fig. 1. Splicing event or subsequent EJC deposition downstream of PTC of TGFβR2 mRNA is enough to trigger NMTR. (A) Schematic diagrams of pRL3-cTGFβR2 with no introns and pRL3-gTGFβR2 with three introns (introns 4–6) of TGFβR2 gene, where gray boxes represent exons of the TGFβR2 gene. A9, PTC, and TAG denote the adenosine mononucleotide repeat where the repeat with ten adenosine residues (A10) was converted to a repeat with nine adenosine residues (A9) due to a frameshift mutation in MSI-H tumors, the premature termination codon generated by a frameshift mutation, and the original translation termination codon of the TGFβR2 gene, respectively. The regions where the oligonucleotides used for semi-quantitative RT-PCR (sqRT-PCR) anneal are indicated by arrows. (B) sqRT-PCR of RLuc-TGFβR2 mRNA and FLuc mRNA. HeLa cells were cotransfected with 1 μg of NMTR reporter plasmid and 1 μg of pGL3-Control to control for variations in transfection and in protein and RNA purification. One day after transfection, total-cell proteins and RNAs were purified. The purified RNAs were analyzed by sqRT-PCR. RLuc-TGFβR2 mRNA was amplified using oligonucleotides #1 and #2. The amount of RLuc-TGFβR2 mRNA was normalized to the amount of FLuc mRNA. Normalized RLuc-cTGFβR2 mRNA was defined as 100%. RT-PCR results obtained in three independent experiments varied by less than 27%. (C) sqRT-PCR of RLuc-TGFβR2 mRNA. As in Fig. 1B, except that RLuc-TGFβR2 mRNA was amplified using oligonucleotides #3 and #4. (D) Translational efficiency of RLuc-cTGFβR2 mRNA and RLuc-gTGFβR2 mRNA. The relative RLuc activity was normalized to the relative level of RLuc mRNA obtained in Fig. 1B. Translational efficiency of RLuc-cTGFβR2 mRNA was defined as 100%. (E) Translational efficiency of RLuc-cTGFβR2 mRNA and RLuc-gTGFβR2 mRNA. The relative RLuc activity was normalized to the relative level of RLuc mRNA obtained in Fig. 1C. Results were obtained from three independent transfections, RNA and protein purifications, sqRT-PCR, and dual luciferase assays. Two-tailed, equal-sample variance Student's *t*-tests were used to determine the statistical significance of differences between the data sets.

(3′UTR) of TGFβR2 gene harboring PTC, which spans from nucleotide immediate downstream of PTC (867th nucleotide numbering from 5′-end of TGFβR2 mRNA) to the 3′-end of TGFβR2 mRNA, was introduced immediately downstream of the translation termination codon of *Renilla* luciferase (RLuc) cDNA in order to generate pRL3-cTGFβR2 and pRL3-gTGFβR2, respectively. In these constructs, the translation termination codon of the RLuc gene would be perceived as a PTC.

We first analyzed relative mRNA levels of RLuc-cTGFβR2 mRNA and RLuc-gTGFβR2 mRNA using semi-quantitative RT-PCR (sqRT-PCR) with total RNAs purified from HeLa cells cotransfected with either pRL3-cTGFβR2 or pRL3-gTGFβR2 reporter plasmid and pGL3-Control plasmid (Fig. 1B–E).

The results of sqRT-PCR performed with specific oligonucleotides #1 and #2, which anneal to the nucleotide sequence within the open reading frame (ORF) of RLuc mRNA, showed that RLuc-gTGFβR2 mRNA was slightly increased in abundance relative to RLuc-cTGFβR2 mRNA (Fig. 1B), rather than decreased due to NMD. Correct splicing of RLuc-gTGFβR2 transcript was further confirmed using oligonucleotides #3 and #4, which amplify the sequence spanning from the 3′-end of exon 4 to the 3′-end of the TGFβR2 gene derived from the vector sequence (Fig. 1A). The results showed that RLuc-gTGFβR2 mRNA was slightly increased in abundance relative to RLuc-cTGFβR2 mRNA (Fig. 1C), consistent with the result obtained in Fig. 1B. Notably, the length of the PCR product from RLuc-gTGFβR2 mRNA was equal to that from RLuc-cTGFβR2 mRNA, indicating that RLuc-gTGFβR2 mRNAs undergo proper splicing. The slight increase of RLuc-gTGFβR2 mRNA abundance (Fig. 1B and C) may be due to the presence of the intron, since splicing or subsequent EJC deposition has been shown to

increase the efficiency of gene expression [4–6]. All results suggest that RLuc-gTGFβR2 mRNA used in this study escapes NMD, even if it contains three introns sufficiently downstream of PTC.

Next, the RLuc activity was monitored and the translation efficiency (normalized RLuc activity per normalized RLuc mRNA) was calculated to assess whether RLuc-gTGFβR2 mRNA was subject to NMTR. The translational efficiency of RLuc-gTGFβR2 mRNA was inhibited by fivefold relative to that of RLuc-cTGFβR2 mRNA (Fig. 1D and E), suggesting that RLuc-gTGFβR2 mRNA is efficiently subjected to NMTR. Our observations indicate that the splicing events or subsequent deposition of EJC downstream of PTC in RLuc-gTGFβR2 mRNA is enough to trigger efficient NMTR.

2.2. eIF4AIII and Y14 are required for NMTR of PTC-containing TGFβR2 mRNAs

To demonstrate that EJC actively functions in NMTR, cellular EJC components or the key NMD factors were downregulated using small interfering RNA (siRNA; Fig. 2). Western blotting showed that the levels of eIF4AIII, Upf1, Y14, and Upf2 were downregulated to 7%, 2%, 10%, and 5% of normal, respectively, where normal is defined as the level in the presence of non-specific control siRNA (Fig. 2A and D). sqRT-PCRs and dual luciferase assays revealed that downregulation of eIF4AIII and Y14 abolished NMTR by 2.4- and 3.0-fold, respectively (Fig. 2C and F), without a significant effect on the abundance of RLuc-TGFβR2 mRNAs (Fig. 2B and E). In contrast, downregulation of Upf1 or Upf2 was of no detectable consequence to NMTR (Fig. 2C and F). Notably, downregulation of eIF4AIII, Upf1, Upf2, or Y14 abrogated the NMD of globin (Gl) mRNA and glutathione peroxidase 1 (GPx1) mRNA harboring PTC by 2–5-fold (Supplementary Fig. S1), suggesting that all tested proteins were downregulated enough to abrogate NMD under our conditions.

The finding that Upf1 is not involved in NMTR was further corroborated by overexpression of the helicase mutant version of Upf1 (Upf1-R844C), which functions as a dominant-negative mutant in NMD (Supplementary Fig. S2). Considering the different requirements for cellular factors in NMD and NMTR, all these results suggest that NMTR is a distinct translational repression mechanism that inhibits the gene expression of PTC-containing mRNAs at the level of translation.

2.3. NMTR occurs after 80S ribosome complex formation

To determine which step of translation of NMTR-targeted mRNA is affected by EJC, we designed RLuc-cTGFβR2 mRNA or RLuc-gTGFβR2 mRNA harboring different types of internal ribosome entry sites (IRESes): encephalomyocarditis virus (EMCV) IRES, hepatitis C virus (HCV) IRES, or cricket paralysis virus (CrPV)

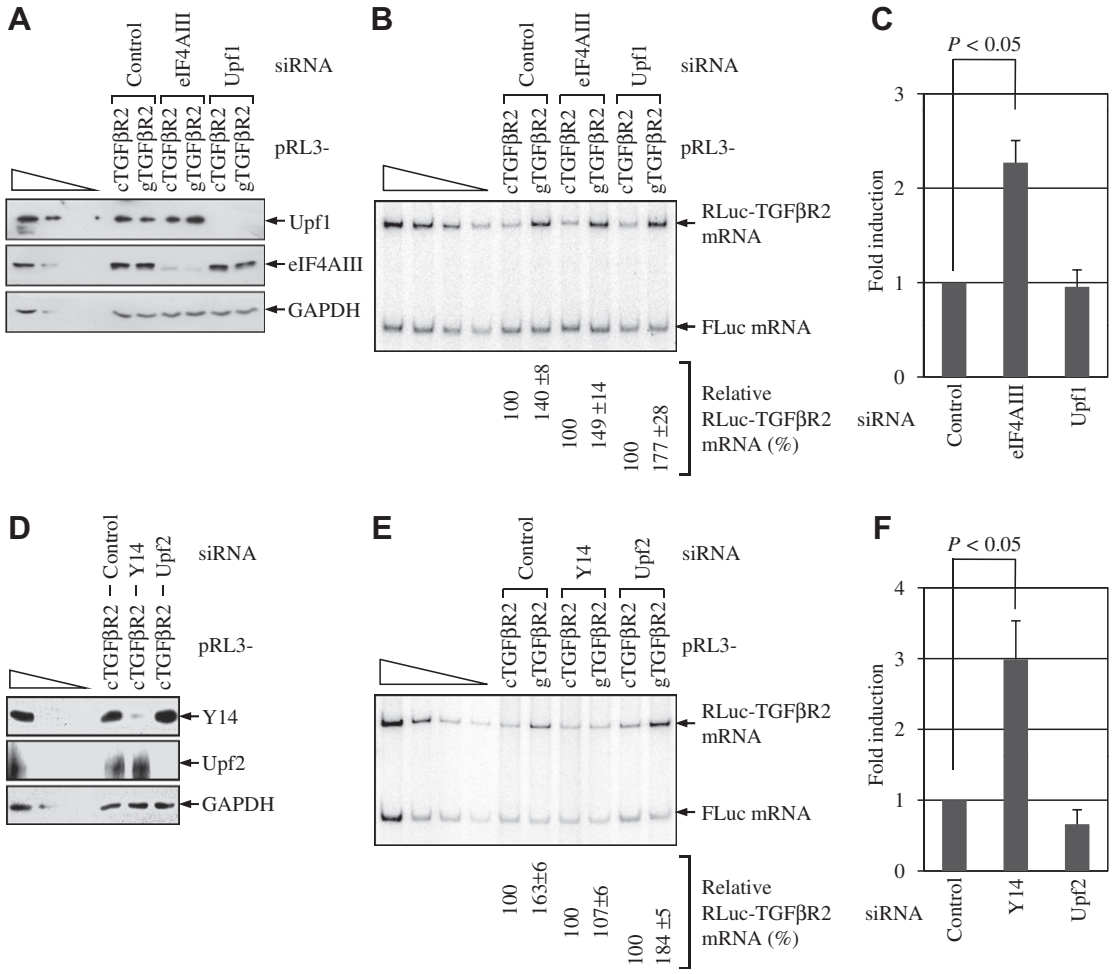

Fig. 2. Downregulation of EJC constituents abrogates NMTR. HeLa cells were transfected with the indicated siRNA. (A and D) Western blotting results to show the selective downregulation. The level of GAPDH controlled for variations in protein purification and sample loading. (B and E) sqRT-PCRs of RLuc-TGFβR2 mRNAs and FLuc mRNAs. sqRT-PCR results from three independent experiments varied by less than 28%. (C and F) Translational efficiency of RLuc-TGFβR2 mRNA in the presence of the indicated siRNA. The relative RLuc activity, which was normalized to FLuc activity, was normalized to the relative level of RLuc mRNA, which was normalized to the level of FLuc mRNA. The ratio of translation efficiency of RLuc-gTGFβR2 mRNA to that of RLuc-cTGFβR2 mRNA in the presence of Control siRNA was defined as 1.

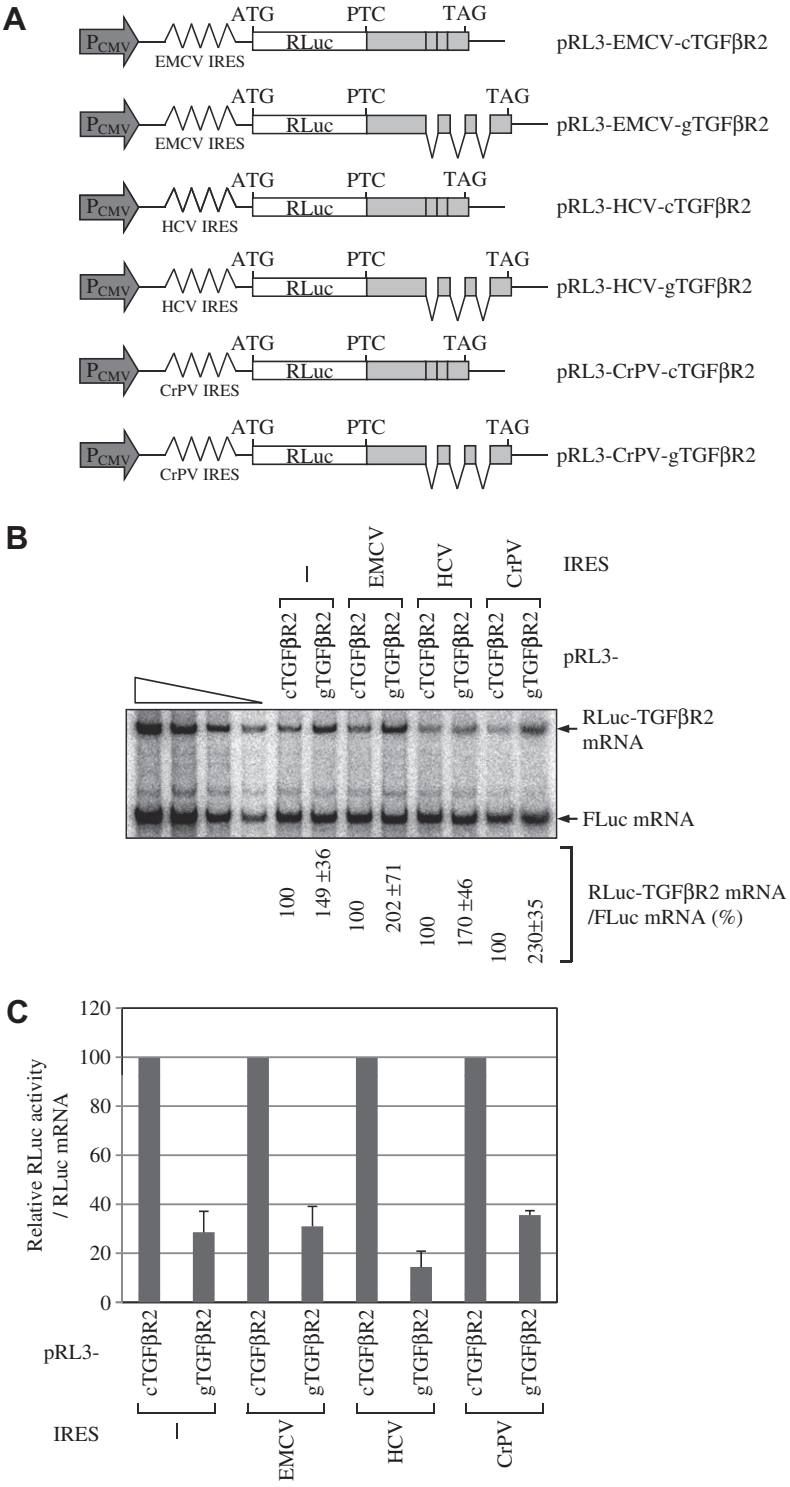

Fig. 3. NMTR occurs after 80S ribosome complex formation. (A) Schematic diagrams of IRES-containing RLuc-cTGFβR2 mRNAs and RLuc-gTGFβR2 mRNAs, where gray boxes represent exons of the TGFβR2 gene. (B) sqRT-PCR of IRES-containing RLuc-cTGFβR2 mRNAs or RLuc-gTGFβR2 mRNAs. HeLa cells were cotransfected with 2 μg of IRES-containing NMTR reporter plasmid and 1 μg of pGL3-Control. The amount of RLuc-cTGFβR2 mRNAs or RLuc-gTGFβR2 mRNAs was normalized to the amount of FLuc mRNA. Each normalized RLuc-cTGFβR2 mRNA was defined as 100%. (C) Translational efficiency of RLuc-cTGFβR2 mRNAs or RLuc-gTGFβR2 mRNAs. The relative RLuc activity was normalized to the relative level of RLuc-TGFβR2 mRNA, which was normalized to the level of FLuc mRNA. Translational efficiency of each RLuc-cTGFβR2 mRNA was defined as 100%.

IRES (Fig. 3A). EMCV IRES, HCV IRES, and CrPV IRES require all of the canonical eukaryotic translation initiation factors (eIFs) except eIF4E, all eIFs except eIF4E and eIF4G, and no eIFs, respectively [7]. We first analyzed mRNA levels and translational efficiencies of IRES-containing RLuc-cTGFβR2 mRNA or RLuc-gTGFβR2 mRNA (Fig. 3B and C). The level of non-IRES- or IRES-containing RLuc-gTGFβR2 mRNA was slightly higher than that of non-IRES- or IRES-containing RLuc-cTGFβR2 mRNA (Fig. 3B), as observed in Fig. 1B and C. In addition, the NMTR efficiency of IRES-containing RLuc-TGFβR2 mRNA (normalized RLuc activity per normalized

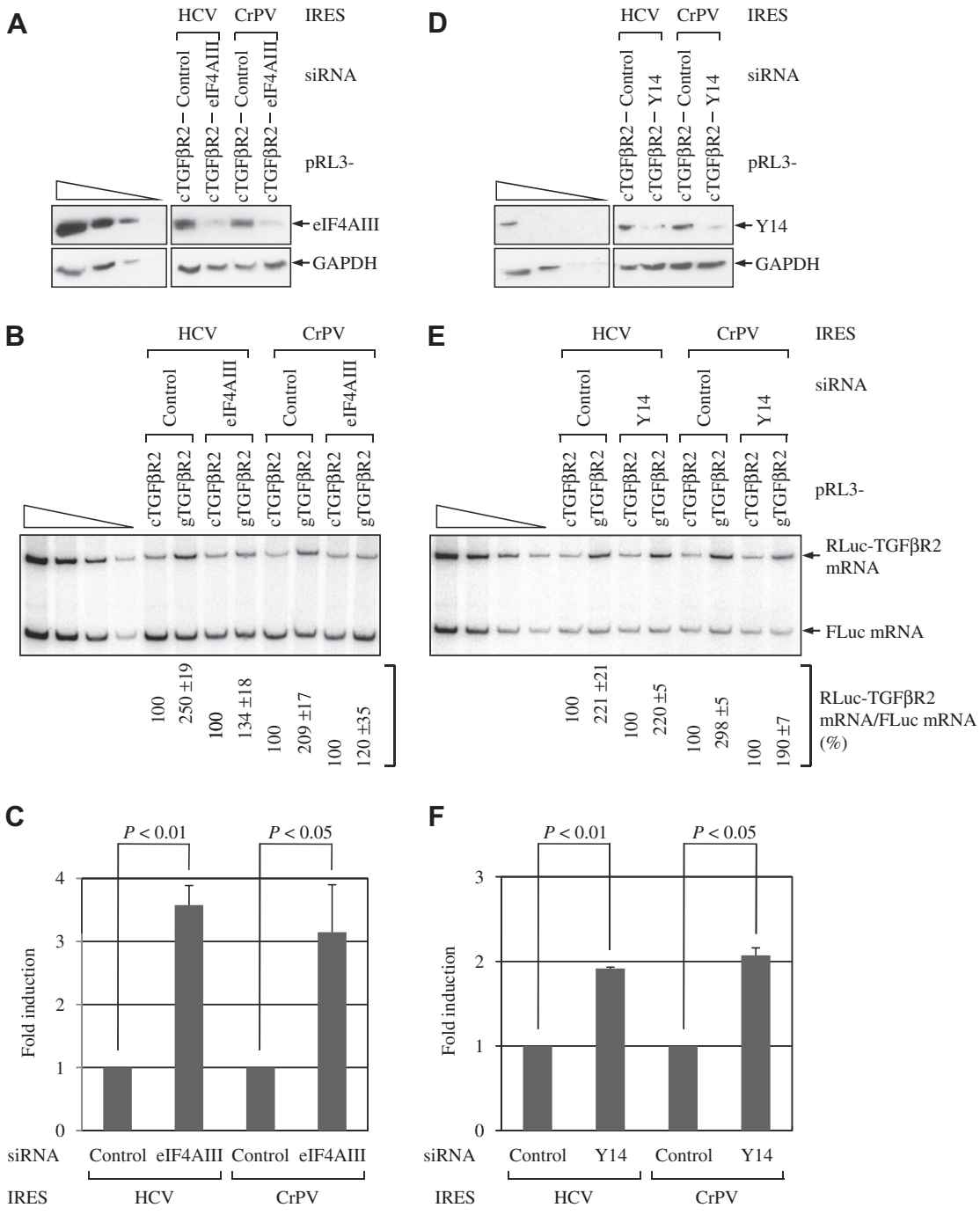

Fig. 4. Downregulation of core EJC constituents abrogates NMTR of HCV IRES- or CrPV IRES-containing RLuc-TGFβR2 mRNAs. HeLa cells were transfected with the indicated siRNA. Two days later, cells were retransfected with 2 μg of IRES-containing NMTR reporter plasmid and 1 μg of pGL3-Control. (A and D) Western blotting results to show selective downregulation. (B and E) sqRT-PCRs of IRES-containing RLuc-TGFβR2 mRNAs and FLuc mRNAs. (C and F) Translation efficiency of IRES-containing RLuc-TGFβR2 mRNAs and FLuc mRNAs. The ratio of translation efficiency of IRES-containing RLuc-gTGFβR2 mRNAs to that of IRES-containing RLuc-cTGFβR2 mRNAs in the presence of control siRNA was defined as 1.

RLuc mRNA) was comparable to that of non-IRES-containing RLuc-TGFβR2 mRNA (Fig. 3C). There was no significant difference of mRNA export efficiency between non-IRES- or IRES-containing RLuc-cTGFβR2 mRNA and RLuc-gTGFβR2 mRNA (Supplementary Fig. S3). These results suggest that NMTR is independent of all eIFs.

Next, we tested whether NMTR of IRES-containing RLuc-gTGFβR2 mRNA is dependent on EJC (Fig. 4). HeLa cells were transfected with eIF4AIII siRNA (Fig. 4A–C), Y14 siRNA (Fig. 4D–F), or a non-specific control siRNA. Two days after siRNA transfection, cells were transiently retransfected with IRES-containing NMTR reporter plasmid and pGL3-control plasmid. Western blotting showed that the levels of endogenous eIF4AIII and Y14 were downregulated to 7% and 10% of normal, respectively (Fig. 4A and D). sqRT-PCR and dual luciferase results revealed that NMTR efficiency of HCV IRES- or CrPV IRES-containing RLuc-TGFβR2 mRNA decreased by 3.6- and 3.1-fold, respectively, upon eIF4AIII downregulation and by 1.9- and 2.1-fold, respectively, upon Y14 downregulation (Fig. 4C and F). Considering the different factor requirements of each IRES, these results suggest that NMTR mediated by EJC occurs after 80S ribosome complex formation.

3. Discussion

Here we demonstrate that EJCs or EJC constituents inhibit translation of NMTR-targeted PTC-containing mRNAs, further expanding the roles of EJCs.

The recognition of PTC by SURF complex, which consists of SMG1, Upf1, and eukaryotic translation termination factors (eRF) 1 and 3, is crucial for efficient NMD in mammalian cells [1,8,9]. Upf1 in SURF complex during translation termination associates with the EJC downstream of the PTC, resulting in the hyperphosphorylation of Upf1. Hyperphosphorylated Upf1 recruits mRNA decay machinery via proline-rich nuclear receptor coregulatory protein 2 (PNRC2) and/or SMG5, SMG6, and SMG7 [10–14], eventually degrading the whole body of PTC-containing mRNAs. Unlike NMD, NMTR does not require the NMD factors, Upf1 and Upf2 (Fig. 2 and reference [3]), suggesting that (i) NMTR may not require the recruitment of SURF complex for the recognition of PTC or (ii) even if SURF complex is recruited to PTC, Upf1 may fail to trigger mRNA decay. Hence, the failure of SURF recruitment or the failure of mRNA degradation mediated by Upf1 during PTC recognition may help PTC-containing mRNAs escape NMD. NMD-escaped mRNAs would still have EJCs downstream of PTC. After PTC recognition and then conformational change of mRNP complex, EJC may trigger NMTR.

The finding that EJC is required for translational repression of NMTR-targeted PTC-containing mRNAs raises some intriguing questions: How are the steps after translation initiation repressed by EJC? Does NMTR-inducing EJC interact with certain translation elongation factors or termination factors? Does EJC play an active role in determining whether PTC-containing mRNAs are subject to either NMD or NMTR? Does EJC loaded onto TGFβR2 mRNA differ in composition from EJCs loaded onto other spliced mRNAs? Future studies will explore these issues.

4. Materials and methods

4.1. Plasmid construction

The details are described in the Supplementary data.

4.2. Cell culture, transfections, and protein and RNA purification

The details are described in the Supplementary data.

4.3. Semi-quantitative reverse-transcription (RT) PCR

Gl mRNA, GPx1 mRNA, and MUP mRNA were amplified as previously described [10,15,16].

RLuc-cTGFβR2 mRNAs and RLuc-gTGFβR2 mRNAs were amplified using specific oligonucleotides: (i) 5′-ATGACTTCGAAAGTTTATG-3′ (oligonucleotide #1, sense) and 5′-TTCAGATTTGATCAACGCA-3′ (oligonucleotide #2, antisense); or (ii) 5′-GGCTAACAGTGGGCAGGTG-3′ (oligonucleotide #3, sense) and 5′-GCCGCCCCGACTCTAGAGCC-3′ (oligonucleotide #4, antisense). FLuc mRNAs transcribed from pGL3-control were amplified using oligonucleotides 5′-CAACACCCCAACATCTTCG-3′ (sense) and 5′-CTTTCCGCCCTTCTTGGCC-3′ (antisense).

IRES-containing TGFβR2 mRNAs were amplified using oligonucleotides #3 and #4.

4.4. Dual luciferase activity assays

Translational efficiency was monitored by luciferase activity, which was measured with the Luciferase assay kit (Promega) according to the manufacturer's instructions.

4.5. Western blotting

The details are described in the Supplementary data.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.01.003.

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